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SHORT COMMUNICATION

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Distal mdx muscle groups exhibiting up-regulation of utrophin and rescue of dystrophin-associated glycoproteins exemplify a protected phenotype in muscular dystrophy

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Abstract Unique unaffected skeletal muscle fibres, unlike necrotic torso and limb muscles, may pave the way for a more detailed understanding of the molecular pathogenesis of inherited neuromuscular disorders and help to develop new treatment strategies for muscular dystrophies. The sparing of extraocular muscle in Duchenne muscular dystrophy is mostly attributed to the special protective properties of extremely fast-twitching small-diameter fibres, but here we show that distal muscles also represent a particular phenotype that is more resistant to necrosis. Immunoblot analysis of membranes isolated from the well established dystrophic animal model mdx shows that, in contrast to dystrophic limb muscles, the toe musculature exhibits an up-regulation of the autosomal dystrophin homologue utrophin and a concomitant rescue of dystrophin-associated glycoproteins. Thus distal mdx muscle groups provide a cellular system that naturally avoids myofibre degeneration which might be useful in the search for naturally occurring compensatory mechanisms in inherited skeletal muscle diseases.

Introduction

Duchenne muscular dystrophy (DMD) represents the most common inherited neuromuscular disorder and eventually affects almost all major muscle groups. Early muscle weakness involves more proximal than distal fibres and initially alters mostly the muscle of the lower extremities and torso. At an intermediate stage, when ambulation is lost, DMD patients can still conduct limited muscle movements with their forearms and hand muscles. Although end-stage DMD also severely weakens hand muscles, a graded dystrophic response of individual groups of skeletal muscle fibres clearly exists during the natural time course of this genetic disorder (Engel et al. 1994). In contrast to a wealth of information published on the sparing of fast-twitching and small-diameter extraocular DMD muscle fibres (Andrade et al. 2000), the adaptive processes involved in the prolonged protection of more distal muscle cells from Ca^{2+}-induced myonecrosis are not well understood.

In order to address this question, we performed a comparative immunoblot analysis of leg and toe muscle specimens from the naturally occurring dystrophic mdx mouse, an established model system of DMD pathology (Allamand and Campbell 2000). The suitability of mdx muscle fibres for research purposes is attested by its wide application in testing novel therapeutic strategies to ameliorate the symptoms of muscular dystrophy (Badalamente and Stracher 2000; Wehling et al. 2001). In analogy to DMD patients, the mdx skeletal muscle fibres are missing the Dp427 isoform of a protein named dystrophin due to primary genetic abnormality (Sicinski et al. 1989). The absence of this membrane cytoskeletal component causes the loss of several sarcolemmal glycoproteins, which is postulated to interrupt the proper linkage between the extracellular matrix and the membrane cytoskeleton, thereby rendering muscle cells more susceptible to necrosis (Ohlendieck 1996). Dystrophin-associated glycoproteins are represented by various isoforms of peripheral proteins termed dystroglycans, sarcoglycans, syntrophins, dystrobrevins and sarcospan (Culligan et al. 1998).

Limb muscle fibres from mdx mice exhibit segmental necrosis, are more susceptible to stretch-induced injury and osmotic shock, and show Ca^{2+}-induced net degradation of muscle proteins (Allamand and Campbell 2000). In both mdx and DMD leg muscle specimens, it has been demonstrated that deficiency in Dp427 does not trigger a general degradation of surface glycoproteins, but specifically causes a reduction in dystrophin-associated glycoproteins (Ohlendieck 1996). Therefore this study measured the relative expression levels of dystroglycans and sarcoglycans in the microsomal membrane fraction,

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which gives a good indication of the dystrophic status of a particular group of skeletal muscle fibres.

Materials and methods

Dystrophin-deficient mdx mice, which are missing the Dp427 dystrophin isoform due to a point mutation in exon 23 (Sicinski et al. 1989), were obtained from Jackson Laboratory (Bar Harbor, Me.). In order to compare the relative expression levels of members of the dystrophin–glycoprotein complex in mdx leg and toe muscles by immunoblotting, established protocols for the isolation of microsomal membranes were employed (Ohlendieck et al. 1991). Since toe muscle fibres represent only a minute amount of tissue, four preparations of skeletal muscles pooled from ten animals each time were used in our analysis. Gel electrophoretic separation, transfer of proteins to nitrocellulose sheets, incubation with primary and secondary antibodies, as well as enhanced chemiluminescence, were carried out by previously optimised methods (Culligan et al. 2001). Antibodies were obtained from Novocastra Laboratories, Newcastle upon Tyne, UK (mAb NCL-43 to β-dystroglycan and mAb DYS-2 to the carboxy-terminus of Dp427), Upstate Biotechnology, Lake Placid, N.Y. (mAb VIA4$_1$ to α-dystroglycan) and Affinity Bioreagents, Golden, Colo. (mAb 20A to the α_2-dihydropyridine receptor). A polyclonal antibody to α-sarcoglycan was characterised as previously described (Culligan et al. 2001) and a polyclonal antibody directed against the carboxy-terminal domain of full-length utrophin was a generous gift from S. Winder (University of Glasgow). Densitometric scanning of immuno-decorated blots was performed on a Molecular Dynamics 300S computing densitometer (Sunnyvale, Calif.) with ImageQuant V3.0 software. Peroxidase-conjugated secondary antibodies were purchased from Boehringer Mannheim (Lewis, East Sussex), Immobilon-P nitrocellulose was from Millipore Corporation (Bedford, Mass.), and all other chemicals were of analytical grade and purchased from Sigma Chemical (Poole, Dorset). Tissue preparation, cryo-sectioning, histochemical staining and microscopy were performed as previously described in detail by Ohlendieck and Campbell (1991).

Results

A well established feature of deficiency in the sub-sarcolemmal component dystrophin is a drastic reduction in numerous muscle surface glycoproteins. However, as illustrated in Fig. 1, our comparative immunoblot analysis demonstrates that this secondary effect on the relative expression of dystrophin-associated glycoproteins does not occur in mdx toe muscle as compared with leg muscle. Although both tissues are lacking the dystrophin isoform Dp427 (Fig. 1a), the expression levels of α-sarcoglycan (Fig. 1e) and α/β-dystroglycan (Fig. 1f, g) are relatively comparable to normal muscle fibre. Importantly, the autosomal dystrophin-homologue named utrophin is up-regulated in mdx toe muscles (Fig. 1b). Laminin expression was not found to be markedly changed in the various muscle specimens studied (Fig. 1d). For control purposes, the relative expression levels of the α_2-subunit of the dihydropyridine receptor are shown (Fig. 1c). Similar amounts of this highly glycosylated marker protein of the muscle cell periphery were found in microsomes isolated from normal, mdx leg and mdx toe muscle homogenates. Thus the observed differences in the relative density of dystrophin-associated glycoproteins be-

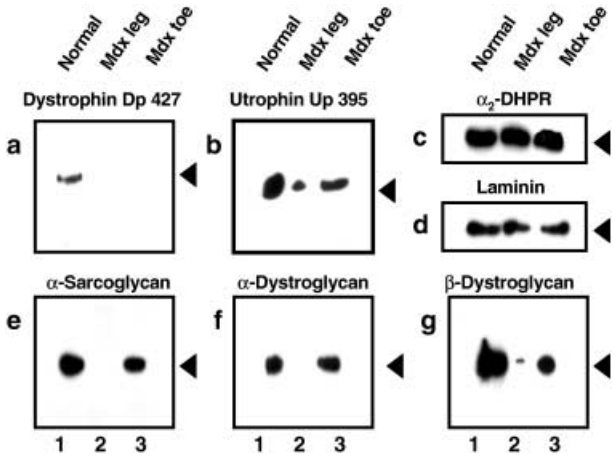

Fig. 1a–g Up-regulation of utrophin and rescue of dystrophin-associated glycoproteins in dystrophin-deficient mdx toe muscle, as demonstrated by comparative immunoblot analysis of microsomes isolated from normal mouse skeletal muscle (*lane 1*), mdx leg muscle (*lane 2*), and mdx toe muscle (*lane 3*). Shown are identical nitrocellulose blots labelled with antibodies to the Dp427 isoform of dystrophin (**a**), the Up395 isoform of utrophin (**b**), the α_2-subunit of the dihydropyridine receptor (α_2-DHPR) (**c**), laminin (**d**), α-sarcoglycan (**e**), α-dystroglycan (**f**), and β-dystroglycan (**g**). In panels (**a**) to (**g**), the expression of α_2-DHPR was used for internal standardisation of the immunoblotting procedure. Immuno-decorated protein bands are marked by *arrow heads*

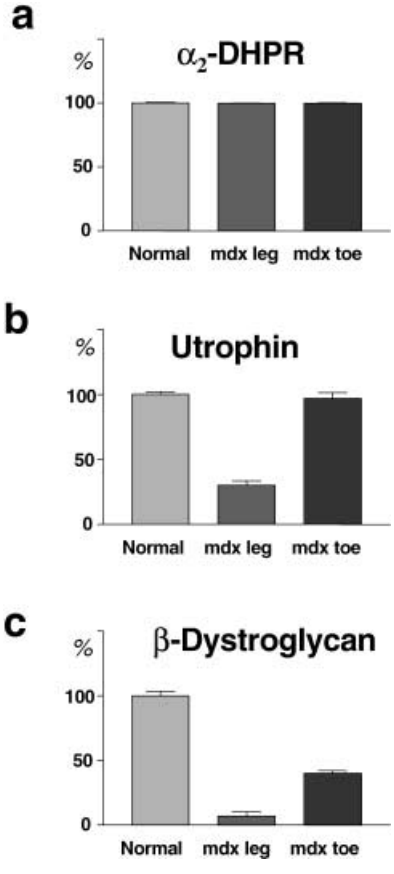

Fig. 2a–c Relative expression of utrophin, dystroglycan and the dihydropyridine receptor in normal, mdx leg and mdx toe muscle fibres. Shown is the densitometric analysis of the immuno-decoration of the α_2-subunit of the dihydropyridine receptor (α_2-DHPR) (**a**), the Up395 isoform of utrophin (**b**) and β-dystroglycan (**c**) (*n*=4)

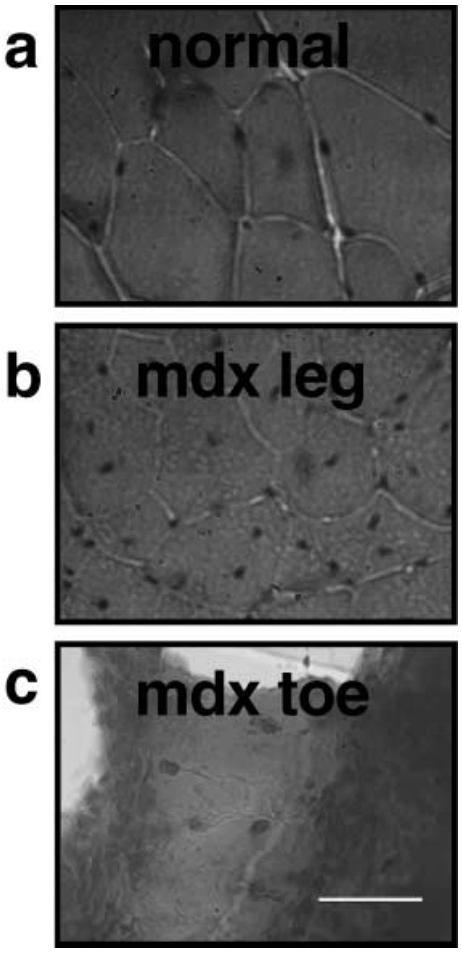

Fig. 3 Histochemical analysis of normal (**a**), mdx leg (**b**) and mdx toe (**c**) muscle fibres. Shown are 10 µm-thick transverse cryosections labelled with haematoxylin and eosin. While normal control muscle fibres (**a**) and mdx toe cells (**c**) exhibit almost exclusively peripheral nuclei, mdx leg muscle fibres show many central nuclei (**b**). Counting of histochemically stained nuclei in 30 fibres revealed approximately 10% peripheral nuclei in mdx leg muscle versus approximately 95% peripheral nuclei in mdx toe cells. Thus, mdx toe muscle, although lacking the dystrophin isoform Dp427, does not appear to show the same degree of degeneration as seen in mdx leg muscle fibres. *Bar* equals 20 µm

tween mdx leg and toe muscle groups is a reliable measurement and is not a result of the subcellular fractionation methodology. As shown in Fig. 2, the densitometric analysis of the immuno-decoration of utrophin and β-dystroglycan shows the up-regulation of utrophin and partial rescue of dystroglycan expression. The immunodecoration intensity of the dihydropyridine receptor appears to be unchanged between normal, mdx leg and mdx toe microsomes (Fig. 2a). This demonstrates that the different expression levels of utrophin and dystrophin-associated glycoproteins in the proximal versus distal limb muscles of the mdx mouse model are not an artefact of the membrane isolation procedure, differential proteolysis, protein solubilisation, electrophoretic separation and/or the immunoblotting technique.

To investigate whether the above described change in the expression levels of utrophin and dystrophin-associated glycoproteins influence the cytopathological status of mdx toe muscle fibres, a comparative histochemical analysis was performed. Haematoxylin and eosin staining clearly revealed the difference between peripheral muscle fibre nuclei in normal tissue (Fig. 3a) versus central muscle fibre nuclei in the mdx leg (Fig. 3b). The observation that mdx toe fibres show mostly peripheral nuclei (Fig. 3c) is in agreement with the notion that the change in the relative density of dystrophin-associated glycoproteins might rescue the dystrophic phenotype in distal muscle groups. This strongly indicates that these muscle cells, although lacking the dystrophin isoform Dp427, do not exhibit the same degree of degeneration seen in mdx leg muscle fibres.

Discussion

In comparison with the established sparing of extraocular muscles, we can show here that a critical factor in the prevention of necrosis in distal muscle fibres appears to be the rescue of dystrophin-associated glycoproteins by a compensatory over-expression of utrophin. In normal skeletal muscle fibres, utrophin is highly enriched at the neuromuscular junction and co-localises with the nicotinic acetylcholine receptor complex (Ohlendieck et al. 1991). However, we were able to show previously that this protein is localised extra-junctionally in mdx fibres (Matsumura et al. 1992), so utrophin might anchor dystrophin-associated glycoproteins in dystrophin-deficient cells. This in turn might reinstate sarcolemmal integrity by at least partially re-linking the extracellular matrix to the actin membrane cystoskeleton and thereby preventing Ca^{2+}-induced protein degradation. That the endogenous up-regulation of utrophin protects the sarcolemmal integrity has been demonstrated by severe dystrophic changes in extraocular mdx-utrn$^{-/-}$ mouse muscle, which lacks both dystrophin and utrophin (Porter et al. 1998).

The finding that distal mdx muscle groups, although deficient in dystrophin, exemplify a protected phenotype, might be of potential significance for both diagnostic and therapeutic purposes. Due to the complexity of the clinical manifestation of inherited muscle diseases, current molecular DMD diagnostics still give occasionally ambiguous results (Miller and Hoffman 1994). Novel strategies for improving the diagnosis of DMD include systematic newborn creatine kinase screening, extended genetic testing covering gene duplications, missense mutations and stop-codon mutations, besides the more common deletions of the DMD gene, as well as carrier testing. Comparing test results for creatine kinase activity between normal muscle, dystrophic mdx leg fibres and protected mdx specimens from the extraocular or toe region might be helpful in refining this diagnostic enzyme procedure. Current therapeutic approaches for DMD include gene therapy (Hartigan-O'Connor and Chamberlain 2000), cell therapy (Partridge and Davies 1995) and pharmacological intervention (Badalamente and Stracher 2000). Important issues for the clinical application of gene therapy are the selection of the opti-

mum delivery system, controlling expression levels without causing immunological and/or cytotoxic side effects and how genes can be sufficiently delivered to all affected cell types (Hartigan-O'Connor and Chamberlain 2000). Potential pharmacological strategies include the drug-induced up-regulation of compensatory molecules such as utrophin, the targeted introduction of protease inhibitors such as carnitine-linked leupeptin in order to inactivate Ca^{2+}-dependent calpain activity, and immunosuppressive therapy. Especially with respect to the idea that up-regulation of utrophin prevents dystrophic changes (Rafael et al. 1998), further investigations into the naturally protected distal mdx muscle groups could help in designing the most favourable gene to be delivered into DMD muscle, whether that be by adenoviral vectors, intravascular delivery of naked DNA or stem cell transfer therapy.

In conclusion, this report demonstrates that distal mdx muscle groups represent unique relatively unaffected skeletal muscle fibres. In contrasting the molecular pathogenesis of necrotic mdx limb muscle with the apparently protected toe fibres, further more refined investigations into these phenotypic differences may pave the way for a more detailed understanding of DMD. Once compensatory cellular mechanisms are better understood, such pathobiochemical knowledge might help in developing new treatment strategies for muscular dystrophies. It remains to be determined whether other cellular factors, besides utrophin up-regulation, such as a lower mechanical stress per unit surface membrane area and/or metabolic differences, are also involved in the sparing of distal mdx muscle fibres.

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